Vol. 138, No. 1, 1986 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
July 16, 1986 Pages 118-124

ISOLATION OF THE CATALYTICALLY COMPETENT SMALL SUBUNIT OF RIBULOSE BISPHOSPHATE CARBOXYLASE/OXYGENASE FROM SPINACH UNDER AN EXTREMELY ALKALINE CONDITION

Aran Incharoensakdi*, Tetsuko Takabe*, Teruhiro Takabe** and T. Akazawa*

*Research Institute for Biochemical Regulation, School of Agriculture, Nagoya University, Chikusa, Nagoya 464, Japan

**Department of Chemistry, Faculty of Science and Technology, Meijo University, Tenpaku, Nagoya 468, Japan

Received May 22, 1986

A method for isolating the small subunit (B) of ribulose-1,5-bisphosphate carboxylase/oxygenase (RuBisCO) from spinach leaf using an alkaline buffer (pH 11.2) in combination with sucrose gradient centrifugation is described. Although the yield of isolated subunit B (ca. 20%) was comparable to that previously described (ca. 25%) using the acid precipitation method [Andrews, T.J. and Lorimer, G.H. (1985) J. Biol. Chem. 260: 4632-4636], the isolated subunit B in this report suffered less denaturation (ca. 30%) as estimated from kinetic analysis of its reassembly with large subunit (A) derived from Aphanothece halophytica. Studies on the kinetic properties of the reassembled enzyme molecules suggested that spinach subunit B does not influence the affinity of the enzyme for substrate CO_2. The catalytic core (A_8) of spinach RuBisCO could not be isolated in the native form.

RuBP carboxylase/oxygenase (RuBisCO) is a bifunctional enzyme, catalyzing the addition of CO_2 as well as O_2 to the substrate RuBP. The structure of RuBisCO from a halophilic cyanobacterium, Aphanothece halophytica, is of a higher plant type consisting of eight copies each of large (A) and small (B) subunits (1). We previously reported that RuBisCO from Aphanothece can be readily dissociated into a catalytic core (A_8) and monomers of subunit B under a condition of low ionic strength (2,3) and that the catalytic core can recombine not only with its own subunit B, but also with subunit B derived from either a cyanobacterium, Synechococcus ACMM 323, or a purple sulfur photosynthetic bacterium, Chromatium vinosum, to produce an enzymatically active RuBisCO molecule (4). The separation of subunit B from

Abbreviation: FPLC, fast protein liquid chromatography; RuBisCO, ribulose-1,5-bisphosphate carboxylase/oxygenase; RuBP, ribulose-1,5-bisphosphate; SDS, sodium dodecyl sulfate.

0006-291X/86 $1.50

the catalytic core so that both entities still remain capable of reassembly without loss of enzyme activity has so far been possible only with a few enzymes of prokaryotic origin (4,5,6). Recently Andrews and Lorimer (7) reported the separation of subunit B from the catalytic core of spinach RuBisCO by mild acid treatment, with the result that subunit B was capable of reassembly with the catalytic core from Synechococcus ACMM 323, whereas the isolated spinach catalytic core was totally denatured. They estimated that 50% of small subunit B thus isolated suffered from denaturation based on the analysis of its reconstitution kinetics. In the present study we report on the separation of subunit B from the catalytic core of spinach RuBisCO by sucrose density gradient centrifugation at pH 11.2. The isolated subunit B was estimated to suffer only 30% denaturation. The assembly of the isolated spinach subunit B after renaturation at neutral pH with the catalytic core from Aphanothece together with some kinetic properties are also presented.

MATERIALS AND METHODS

Native RuBisCO, the catalytic core and subunit B of Aphanothece halophytica were prepared as described previously (8). RuBisCO from Chromatium vinosum was purified according to the method of Brown et al (9) and the catalytic core and subunit B were prepared as described in a previous report (4).

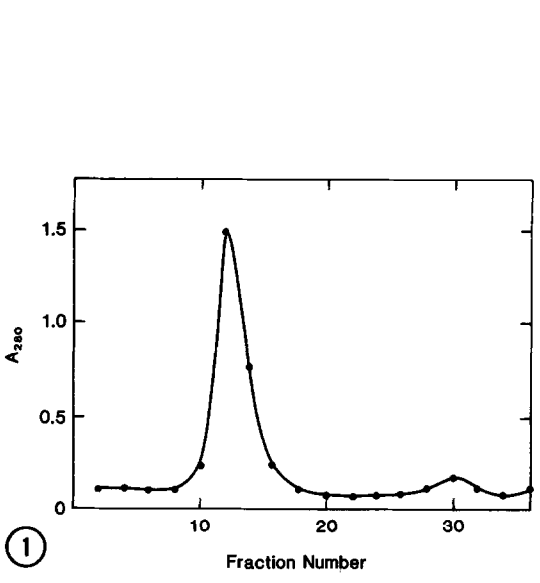
Spinach RuBisCO was purified by the method reported by Hall and Tolbert (10). To separate subunits of spinach RuBisCO, about 10 mg of purified enzyme was dissolved in 3 ml of 50 mM sodium phosphate-NaOH buffer, pH 11.2, and dialyzed in the same buffer for 4 h at 4°C. The dialyzate (1 ml) was layered on a linear gradient solution of sucrose (0.2-0.8 M) dissolved in the dialysis buffer and centrifuged in a Beckman VTi 50 rotor at 242,000 x g (r_{max}) for 2.5 h at 4°C. Fractions collected from the bottom were monitored at A_{280}. Protein peak fractions were pooled and precipitated with 80% ammonium sulfate. The protein pellets were suspended in a small volume of 10 mM sodium phosphate buffer, pH 7.6, containing 1 mM EDTA and dialyzed for 3 h at 4°C before being used for reconstitution experiments. Pellets derived from fast-sedimenting fractions could not be dissolved in a neutral buffer without the aid of denaturing agents.

Cross-contamination of subunits was evaluated by FPLC analysis using a prepacked Superose 12 column from Pharmacia. Enzyme samples were treated with 1% (w/v) SDS and 1% (v/v) 2-mercaptoethanol at 100°C for 5 min. They were then injected (typically 100 µl) into the column preequilibrated with 50 mM Tris-HCl buffer, pH 7.5, containing 0.1% (w/v) SDS and 0.1% (v/v) 2-mercaptoethanol. Protein, monitored at A_{280}, was eluted with the same buffer at a flow rate of 0.5 ml/min with an inlet pressure up to 2.8 M Pa.

RuBP caboxylase activity was assayed by the radiometric method reported previously (8). Protein content was analyzed by a sensitive dye-binding method (11) modified by Bio-Rad Laboratories using bovine serum albumin as standard. Molecular weights of subunit A and B were estimated by SDS-polyacrylamide gel electrophoresis according to Laemmli (12).

RESULTS AND DISCUSSION

The protein profile of the spinach RuBisCO subjected to sucrose density gradient (pH 11.2) centrifugation is shown in Fig. 1. Two protein peaks were found. The fast and the slow sedimenting fractions were subjected to SDS-FPLC analysis using a prepacked Superose 12 column from Pharmacia. The slow sedimenting fraction contained small subunit B without contamination of large subunit A as shown in Fig. 2C, whereas the fast sedimenting fractions contained subunit A and an appreciable amount of subunit B (Fig. 2B) (ca. 80% of subunit B was associated with subunit A on a molar basis). By using purified spinach RuBisCO (Fig. 2A), the two peaks eluted at 10 and 23 min were identified as subunits A and B, respectively. The extent of dissociation of subunit B from the catalytic core during sucrose gradient centrifugation did not appear to be significantly affected by either temperature or salt.



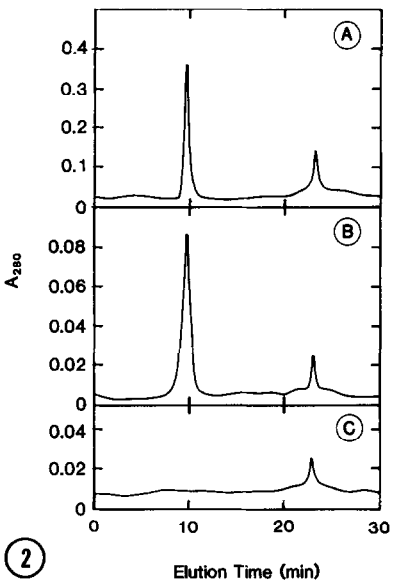


Fig. 1. Sucrose density gradient centrifugation of spinach RuBisCO at pH 11.2. The gradient after centrifugation at 4°C was fractionated (1.1 ml) from the bottom and absorbance at 280 nm was measured.

Fig. 2. FPLC of spinach RuBisCO (A), catalytic core (B), and subunit B (C) in the presence of SDS. Spinach RuBisCO (200 µg), catalytic core (35 µg) and subunit B (1.5 µg) treated with 1% (w/v) SDS and 1% (v/v) 2-mercaptoethanol were injected into a prepacked Superose 12 column (Pharmacia). The elution buffer was 50 mM Tris-HCl buffer, pH 7.0, containing 0.1% (w/v) SDS and 0.1% (v/v) 2-mercaptoethanol with a flow rate of 0.5 ml/min. Note the difference in the scale of A_{280} in panel A.

Table 1

Kinetic Parameters of the Reassembled Enzymes

Reassembled Enzyme	K_D (nM)	V (μmol/min/mgA)	b
*A^aB^a	84 + 31	4.89 + 0.44	0.90 + 0.13
A^aB^S	3684 + 331	2.34 + 0.63	0.70 + 0.15

K_D, V and b (fraction of undenatured subunit B) were estimated as described in Fig. 3. The estimates are presented together with standard deviations (+ S.D.).

*Data were taken from reference 4 where the catalytic core and subunit B were isolated by sucrose density gradient centrifugation under a condition of low ionic strength.

Previously it has been reported that subunits of spinach RuBisCOs are separable under an alkaline pH around 9.0 in the presence of p-chloromercuribenzoate (13) and also by Sephadex G-200 gel filtration at pH 11.2 (14), but in neither case was the assessment of the obtainable subunit B thoroughly studied. The isolated subunit B obtained by Nishimura et al (13) was able to reassociate with the catalytic core but the original enzyme activity could not be restored. This result suggested that some denaturation of either the catalytic core or subunit B might have occurred during the separation process. The present report provides a method of separating subunit B in a purified form with the advantage that the extent of the denaturation of subunit B was much reduced (ca. 30% denaturation as shown in Table 1). It is also noted that the yield of the isolated subunit B was about 20%, comparable to that obtained by the acid precipitation method (25%, ref. 7).

The enhancement of RuBP carboxylase activity was observed when increasing amount of subunit B from spinach RuBisCO was added to a fixed amount of catalytic core from Aphanothece (Fig. 3). The catalytic core from Chromatium, on the other hand, could not reassemble with spinach subunit B. This is in agreement with a previous report of the unsuccessful reassembly of Chromatium catalytic core with tobacco subunit B (6).

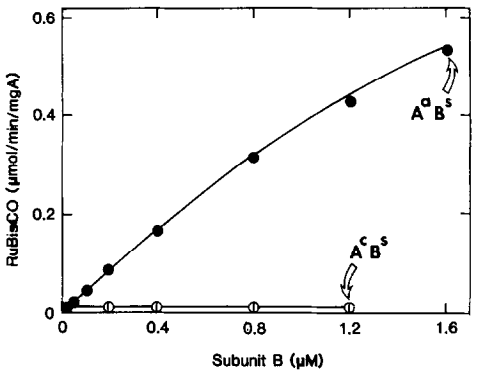


Fig. 3. Reassembly of Aphanothece and Chromatium catalytic cores with spinach subunit B. Catalytic cores from Aphanothece and Chromatium (100 nM subunit A in both cases) were mixed with increasing concentrations of subunit B from spinach. Activity was measured by preincubating the mixture in a total volume of 225 μl containing 100 mM Tris-HCl buffer, pH 7.8, 0.1 mg/ml BSA, 20 mM $MgCl_2$, 50 mM $NaH^{14}CO_3$ (4 Ci/mol) for 15 min at 25°C. The 10 min-reaction was initiated by the addition of 25 μl of 10 mM RuBP and stopped by the addition of 50 μl of acetic acid. Data were analyzed according to eq. 1 described in the text by means of nonlinear regression analysis with the aid of the LSANLS/D program developed by the Computer Center, Nagoya University and best fitted lines were obtained. Superscripts a, c and s denote the organisms, i.e., Aphanothece, Chromatium and spinach, respectively.

The kinetics of the association of the catalytic core with subunit B, producing homologous (A^aB^a) or heterologous (A^aB^S) enzymes, is analyzed by eq. 1 which is slightly modified from the one previously reported by Andrews and Lorimer (7).

$$v = V\left\{bBt + At + K_D - [\,(bBt + At + K_D)^2 - 4\,At.bBt\,]^{1/2}\right\}/\,2At \qquad (1)$$

The values of K_D (binding constant between subunit A and B), V (specific activity at saturated concentration of subunit B), and b (fraction of the undenatured subunit B) are presented in Table 1.

A^aB^S exhibited about half of the specific activity of A^aB^a. The binding of B^S to A^a occurred much less tightly than the binding of B^a to A^a, at least with two order of magnitude difference in K_D. The loose binding of the heterologous enzyme has been previously observed (catalytic core from Aphanothece and subunit B from Synechococcus) with the K_D value of 2.5 μM (4). The most important result, however, is that most of isolated subunit B was mainly undenatured as evaluated by its ability to combine with the catalytic

core to produce a catalytically competent enzyme molecule. The extent of denaturation of isolated subunit B from spinach RuBisCO using acid precipitation method was reported to be about 50% (7). The lower denaturation rate in the present study probably results from the less damaging effect of alkaline pH as well as the protective effect of sucrose during the centrifugation. It is interesting to note also that subunit B isolated from Aphanothece by sucrose density gradient centrifugation under a condition of low ionic strength was marginally denatured (ca. 10 %).

We also studied the effect of subunit B on the kinetic properties of RuBisCO using the heterologously reassembled enzyme, A^aB^S in comparison with the homologous enzyme A^aB^a. This is feasible only when there is a marked difference in the kinetic properties between the two parent RuBisCO molecules [e.g. $K_m(CO_2)$]. Although it was found that the K_m (CO_2) value of Aphanothece RuBisCO is over 10 times higher than that of spinach RuBisCO, no apparent difference was observed in K_m (CO_2) value between A^aB^a and A^aB^S enzymes (data not shown). The results suggest that the affinity of the catalytic core for the substrate CO_2 is not largely influenced by subunit B. The presence of subunit B only influences the maximum velocity of the reassembled enzyme; an approximately two-fold increase was observed with A^aB^a comparing to A^aB^S.

So far the role of subunit B in the catalysis of RuBisCO has not been definitely assigned, although recent work in our laboratory has implicated subunit B as a stabilizer of the activated enzyme molecule (15). Now that isolated subunit B from spinach is available in a less denatured form, it may be useful for the investigation of its role in enzyme catalysis as well as the mechanism of its assembly with the large subunit.

ACKNOWLEDGEMENTS

We thank Dr. A. Renwick and Dr. M.T. Siva-Jothy for critical reading of the manuscript. This is paper No. 69 in the series "Structure and Function of Chloroplast Proteins". This research was financially supported by a research grant from the Ministry of Education, Science and Culture of Japan (60560089). A.I. is a recipient of the scholarship from the Japanese government and is on leave from Department of Biochemistry, Faculty of Science, Chulalongkorn University, Bangkok, Thailand.

REFERENCES

1) Akazawa, T. (1979) In Encyclopedia of Plant Physiology, New Ser., Vol. 6. Edited by M. Gibbs and E. Latzko. pp. 208-229. Springer-Verlag, Berlin.
2) Asami, S., Takabe, T., Akazawa, T., and Codd, G.A. (1983) Arch. Biochem. Biophys. 225:713-721.
3) Takabe, T., Rai, A.K., and Akazawa, T. (1984) Arch. Biochem. Biophys. 229: 202-211.
4) Incharoensakdi, A., Takabe, T., Takabe, T., and Akazawa, T. (1985) Biochem. Biophys. Res. Commun. 126:698-704.
5) Andrews, T.J., and Ballment, B. (1983) J. Biol. Chem. 258:7514-7518.
6) Jordan, D.B., and Chollet, R. (1985) Arch. Biochem. Biophys. 236:487-496.
7) Andrews, T.J., and Lorimer, G.H. (1985) J. Biol. Chem. 260:4632-4636.
8) Incharoensakdi, A., Takabe, T., and Akazawa, T. (1985) Arch. Biochem. Biophys. 237:445-453.
9) Brown, H.M., Bowman, L.H., and Chollet, R. (1981) FEMS Microbiol. Lett. 12, 105-109.
10) Hall, N.P., and Tolbert, N.E. (1978) FEBS Lett. 96:167-169.
11) Bradford, M.M. (1976) Anal. Biochem. 72:248-254.
12) Laemmli, U.K. (1970) Nature 227:680-685.
13) Nishimura, M., and Akazawa, T. (1974) J. Biochem. (Tokyo) 76, 169-176.
14) Kawashima, N., and Wildman, S.G. (1971) Biochim. Biophys. Acta 229, 749-760.
15) Incharoensakdi, A., Takabe, T., and Akazawa, T. (1986) Arch. Biochem. Biophys. (in press).